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(21) International Application Number: PCT/US91/04384 (22) International Filing Date: 19 June 1991 (19.06.91) (30) Priority data: 541,108 20 June 1990 (20.06.90) US (60) Parent Application or Grant (63) Related by Continuation US 541,108 (CIP) Filed on 20 June 1990 (20.06.90) (71) Applicant (for all designated States except US): AFFYMAX TECHNOLOGIES N.V. [NL/NL]; De Ruyderkade 62, Curaçao (AN). (72) Inventors; and (75) Inventors/Applicants (for US only) : DOWER, William, J. [US/US]; 761 Partridge Avenue, Menlo Park, CA 94025 (US). CWIRLA, Steven, E. [US/US]; 765 San Antonio Road, #64, Palo Alto, CA 94303 (US). BARRETT, Ronald, W. [US/US]; 562 Carlise Way, Sunnyvale, CA 94087 (US).		(74) Agent: SMITH, William, M.; Townsend and Townsend, One Market Plaza, 2000 Steuart Tower, San Francisco, CA 94105 (US). (81) Designated States: AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CI (OAPI patent), CM (OAPI patent), DE, DE (European patent), DK, DK (European patent), ES, ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), GN (OAPI patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL, NL (European patent), NO, PL, RO, SD, SE, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent), US. Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: PEPTIDE LIBRARY AND SCREENING SYSTEMS		
(57) Abstract Peptide which bind to selected receptors are identified by screening libraries which encode a random or controlled collection of amino acids. Peptides encoded by the libraries are expressed as fusion proteins of bacteriophage coat proteins, and bacteriophage are then screened against the receptors of interest. Peptides having a wide variety of uses, such as therapeutic or diagnostic reagents, may thus be identified without any prior information on the structure of the expected ligand or receptor.		

PEPTIDE LIBRARY AND SCREENING SYSTEMS

5

Field of the Invention

10 The present invention relates generally to
methods for selecting peptide ligands to receptor
molecules of interest and, more particularly, to methods
for generating and screening large peptide libraries for
peptides with desired binding characteristics.

15

Background of the Invention

20 As molecular biology has helped to define
regions of proteins that contribute to a particular
biological activity, it has become desirable to
synthesize short peptides to mimic (or inhibit) those
activities. Many of the disadvantages encountered in
therapeutic, diagnostic and industrial settings with
purified proteins, or those produced by recombinant
25 means, could easily be avoided by short synthetic
peptides. For instance, synthetic peptides offer
advantages of specificity, convenience of sample or bulk
preparation, lower relative cost, high degree of purity,
and long shelf-life.

30

peptides, the technology remains, to a large extent, a
laboratory tool. Precise sequence and binding data are
not available for most proteins of significant medical,
agricultural or industrial interest. Even when the
35 sequence of a protein is known, the process of

preselected receptor. In certain aspects the methods generally comprise constructing a bacteriophage expression vector which comprises an oligonucleotide library of at least about 10^6 members which encode the peptides. The library member is joined in reading frame to the 5' region of a nucleotide sequence encoding an outer structural protein of the bacteriophage. Appropriate host cells are transformed with the expression vectors, generally by electroporation, and the transformed cells cultivated under conditions suitable for expression and assembly of bacteriophage. Using an affinity screening process, bacteriophage library members are contacted with the preselected receptor under conditions conducive to specific peptide-receptor binding, and bacteriophage whose coat proteins have peptides which bind the receptor molecule are selected. The nucleotide sequence which encodes the peptide on the selected phage may then be determined. By repeating the affinity selection process one or more times, the peptides of interest may be enriched. By increasing the stringency of the selection, e.g., by reducing the valency of the peptide-phage interaction towards substantial monovalency, peptides of increasingly higher affinity can be identified.

In another aspect the methods are concerned with expression vectors having the oligonucleotide library members joined in reading frame with a nucleotide sequence to encode a fusion protein, wherein the library member represents the 5' member of the fusion and the 3' member comprises at least a portion of an outer structural protein of the bacteriophage. The first residue of the peptide encoded by the library member may be at the 5'-terminus of the sequence encoding the phage coat protein. In preferred embodiments, where phage proteins are initially expressed as preproteins and then processed by the host cell to a mature protein, the library members are inserted so as to leave the peptide

and a 2 (gly) codon spacer, was ligated to the vector and electro-transformed into E. coli.

Fig. 2 depicts the amino acid sequences (deduced from DNA sequence) of the N-terminal hexapeptides on pIII of infectious phage randomly selected from the library. Sequences begin at the signal peptidase site. Single letter code for amino acids is A (Ala), C (Cys), D (Asp), E (Glu), F (Phe), G (Gly), H (His), I (Ile), K (Lys), L (Leu), M (Met), N (Asn), P (Pro), Q (Gln), R (Arg), S (Ser), T (Thr), V (Val), W (Trp), Y (Tyr).

Fig. 3 illustrates the composite DNA sequence of the variable region of pools of (A) infectious phage from the library, and (B) phage recovered from 1, 2, or 3 rounds of panning on mAB 3E7. Phage were amplified as tetracycline resistant colonies and DNA from a pool of phage derived from several thousand of these colonies was isolated and sequenced. The area of the sequencing gel corresponding to the cloning site in geneIII is displayed. A sequencing primer was annealed to the phage DNA ~40 bases to the 3' side of the cloning site. The actual readout of the gel is the sequence complementary to the coding strand. For clarity of codon identification, the lanes may be read as C, T, A, G, left to right and 5' to 3', top to bottom, to identify the sequence of the coding (+) strand.

Fig. 4 shows the amino acid sequences (deduced from DNA sequence) of the N-terminal peptides of pIII of 51 phage isolated by three rounds of panning on mAB 3E7.

Fig. 5 illustrates the results of phage sandwich ELISAs for YGGFL- and YAGFAQ-phage with biotinylated monoclonal antibody 3E7 IgG (Fig. 5A) or 3E7 Fab fragments (Fig. 5B) immobilized at maximal density on streptavidin coated wells and labeled polyclonal anti-phage antibodies to detect bound phage.

Fig. 6 illustrates the results of phage sandwich ELISAs which compare the effect of 3E7 Fab

act as a signal or messenger, to stimulate or inhibit cellular activity, and the like.

The number of possible receptor molecules for which peptide ligands may be identified by means of the present invention is virtually unlimited. For example, the receptor molecule may be an antibody (or a binding portion thereof). The antigen to which the antibody binds may be known and perhaps even sequenced, in which case the invention may be used to map epitopes of the antigen. If the antigen is unknown, such as with certain autoimmune diseases, for example, sera or other fluids from patients with the disease can be used in the present methods to identify peptides, and consequently the antigen which elicits the autoimmune response. It is also possible using these methods to tailor a peptide to fit a particular individual's disease. Once a peptide has been identified it may itself serve as, or provide the basis for, the development of a vaccine, a therapeutic agent, a diagnostic reagent, etc.

The present invention can identify peptide ligands for a wide variety of substances in addition to antibodies. These include, by way of example and not limitation, growth factors, hormones, enzymes, interferons, interleukins, intracellular and intercellular messengers, lectins, cellular adhesion molecules and the like, as well as the ligands for the corresponding receptors of the aforementioned molecules. It will be recognized that peptide ligands may also be identified by the present invention for molecules which are not peptides or proteins, e.g., carbohydrates, non-protein organic compounds, metals, etc. Thus, although antibodies are widely available and conveniently manipulated, they are merely representative of receptor molecules for which peptide ligands can be identified by means of the present invention.

An oligonucleotide library, prepared according to the criteria as described herein, is inserted in an

The library is constructed by cloning an oligonucleotide which contains the variable region of library members (and any spacers, framework determinants, etc. as discussed below) into the selected cloning site.

5 Using known recombinant DNA techniques (see generally, Sambrooke et al., Molecular Cloning, A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989, incorporated by reference herein), an oligonucleotide may be constructed which, inter alia,

10 removes unwanted restriction sites and adds desired ones, reconstructs the correct portions of any sequences which have been removed (such as a correct signal peptidase site, for example), inserts the spacer conserved or framework residues, if any, and corrects the translation

15 frame (if necessary) to produce active, infective phage. The central portion of the oligonucleotide will generally contain one or more of the variable region domain(s) and the spacer or framework residues. The sequences are ultimately expressed as peptides (with or without spacer

20 or framework residues) fused to or in the N-terminus of the mature coat protein on the outer, accessible surface of the assembled bacteriophage particles.

The variable region domain of the oligonucleotide comprises the source of the library. The

25 size of the library will vary according to the number of variable codons, and hence the size of the peptides, which are desired. Generally the library will be at least about 10^6 members, usually at least 10^7 , and typically 10^8 or more members. To generate the collection

30 of oligonucleotides which forms a series of codons encoding a random collection of amino acids and which is ultimately cloned into the vector, a codon motif is used, such as $(NNK)_x$, where N may be A, C, G, or T (nominally equimolar), K is G or T (nominally equimolar), and x is

35 typically up to about 5, 6, 7, or 8 or more, thereby producing libraries of penta-, hexa-, hepta-, and octa-peptides or more. The third position may also be G or C,

ratios will usually be approximately equimolar, but may be a controlled unequal ratio to obtain the over- to under-representation of certain amino acids coded for by the degenerate oligonucleotide collection. The condensation of the trimers to form the oligocodons is done essentially as described for conventional synthesis employing activated mononucleosides as building blocks. See generally, Atkinson and Smith, Oligonucleotide Synthesis, M.J. Gait, ed. p35-82 (1984). Thus, this procedure generates a population of oligonucleotides for cloning that is capable of encoding an equal distribution (or a controlled unequal distribution) of the possible peptide sequences. This approach may be especially useful in generating longer peptide sequences, since the range of bias produced by the $(NNK)_6$ motif increases by three-fold with each additional amino acid residue.

When the codon motif is $(NNK)_x$, as defined above, and when x equals 8, there are 2.6×10^{10} possible octa-peptides. A library containing most of the octa-peptides may be difficult to produce. Thus, a sampling of the octa-peptides may be accomplished by constructing a subset library using of about .1%, and up to as much as 1%, 5% or 10% of the possible sequences, which subset of recombinant bacteriophage particles is then screened. As the library size increases, smaller percentages are acceptable. If desired, to extend the diversity of a subset library the recovered phage subset may be subjected to mutagenesis and then subjected to subsequent rounds of screening. This mutagenesis step may be accomplished in two general ways: the variable region of the recovered phage may be mutagenized, or additional variable amino acids may be added to the regions adjoining the initial variable sequences.

A variety of techniques can be used in the present invention to diversify a peptide library or to diversify around peptides found in early rounds of panning to have sufficient binding activity. In one

general mutagenesis method is described in detail in Myers, et al., Nucl. Acids Res. 13:3131-3145 (1985), Myers et al., Science 229:242-246 (1985), and Myers, Current Protocols in Molecular Biology Vol I, 8.3.1 - 8.3.6, F. Ausubel, et al., eds, J. Wiley and Sons, New York (1989), each of which are incorporated herein by reference.

In the second general approach, that of adding additional amino acids to a peptide or peptides found to be active, a variety of methods are available. In one, the sequences of peptides selected in early panning are determined individually and new oligonucleotides, incorporating the determined sequence and an adjoining degenerate sequence, are synthesized. These are then cloned to produce a secondary library.

In another approach which adds a second variable region to a pool of peptide-bearing phage, a restriction site is installed next to the primary variable region. Preferably, the enzyme should cut outside of its recognition sequence, such as BspMI which cuts leaving a four base 5' overhang, four bases to the 3' side of the recognition site. Thus, the recognition site may be placed four bases from the primary degenerate region. To insert a second variable region, the pool of phage DNA is digested and blunt-ended by filling in the overhang with Klenow fragment. Double-stranded, blunt-ended, degenerately synthesized oligonucleotide is then ligated into this site to produce a second variable region juxtaposed to the primary variable region. This secondary library is then amplified and screened as before.

While in some instances it may be appropriate to synthesize peptides having contiguous variable regions to bind certain receptors, in other cases it may be desirable to provide peptides having two or more regions of diversity separated by spacer residues. For example, the variable regions may be separated by spacers which

serve to mimic a cyclic peptide. Of course, those skilled in the art will appreciate that various other types of covalent linkages for cyclization may also be accomplished.

5 The spacer residues described above can also be situated on either or both ends of the variable nucleotide region. For instance, a cyclic peptide may be accomplished without an intervening spacer, by having a Cys residue on both ends of the peptide. As above,
10 flexible spacers, e.g., oligo-glycine, may facilitate interaction of the peptide with the selected receptors. Alternatively, rigid spacers may allow the peptide to be presented as if on the end of a rigid arm, where the number of residues, e.g., Pro, determines not only the
15 length of the arm but also the direction for the arm in which the peptide is oriented. Hydrophilic spacers, made up of charged and/or uncharged hydrophilic amino acids, (e.g., Thr, His, Asn, Gln, Arg, Glu, Asp, Met, Lys, etc.), or hydrophobic spacers of hydrophobic amino acids
20 (e.g., Phe, Leu, Ile, Gly, Val, Ala, etc.) may be used to present the peptides to binding sites with a variety of local environments.

Unless modified during or after synthesis by the translation machinery, recombinant peptide libraries
25 consist of sequences of the 20 normal L-amino acids. While the available structural diversity for such a library is large, additional diversity can be introduced by a variety of means, such as chemical modifications of the amino acids.

30 For example, as one source of added diversity a peptide library of the invention can have its carboxy terminal amidated. Carboxy terminal amidation is necessary to the activity of many naturally occurring bioactive peptides. This modification occurs in vivo
35 through cleavage of the N-C bond of a carboxy terminal Gly residue in a two-step reaction catalyzed by the enzymes peptidylglycine alpha-amidation monooxygenase

terminus exposed on the extracellular side of the membrane. In this embodiment the variable oligonucleotides region, having a stop codon in the last position, is inserted in the 3' end of a sequence which encodes C terminus exposed protein, or at least a portion of the protein that is responsible for the C-terminus out orientation. The transferrin receptor protein is an example of one such protein. This receptor has been cloned and sequenced, as reported in McClelland et al., Cell 39:267-274 (1984), incorporated herein by reference. An internal transmembrane segment of the transferrin receptor serves to orient the protein with its carboxy terminus out. When the cDNA is expressed, typically in eucaryotic cells, the random peptides are located extracellularly, having their amino terminus fused to the transferrin receptor and with a free carboxy terminus.

For carboxy terminal peptide libraries, a COS cell expression cloning system can also be used and may

be preferred in some circumstances. COS cells are transfected with a variable nucleotide library contained in an expression plasmid that replicates and produces mRNA extrachromosomally when transfected into COS cells. Transfected cells bearing the random peptides are selected on immobilized ligand or cells which bear a binding protein, and the plasmid is isolated (rescued) from the selected cells. The plasmid is then amplified and used to transfect COS cells for a second round of screening. Because the random oligonucleotides are inserted directly into the expression plasmid, much larger libraries (i.e., total number of novel peptides) are constructed. Of course, for each round of panning the plasmid needs to be rescued from the COS cells, transfected into bacteria for amplification, re-isolated and transfected back into COS cells.

Other expression systems for carboxy terminal amidation of peptides of the invention can also be used. For example, the variable oligonucleotide sequences are

polyvalent interactions with an immobilized target receptor. Another advantage of the baculovirus system is that, similar to the peptide on phage method, infectivity is exploited to amplify virus which is selected by the panning procedure. During the series of pannings, the DNA does not need to be isolated and used for subsequent transfections of cells.

Other expression systems can be employed in the present invention. As eucaryotic signal sequences are operable in yeast and bacteria, proteins with a carboxy terminus out orientation, such as the transferrin receptor, can be appropriately expressed and oriented in yeast or bacteria. The use of yeast or bacteria allows large libraries and avoids potential problems associated with amplification.

Other modifications found in naturally occurring peptides and proteins can be introduced into the libraries to provide additional diversity and to contribute to a desired biological activity. For example, the variable region library can be provided with codons which code for amino acid residues involved in phosphorylation, glycosylation, sulfation, isoprenylation (or the addition of other lipids), etc. Modifications not catalyzed by naturally occurring enzymes can be introduced by chemical means (under relatively mild conditions) or through the action of, e.g., catalytic antibodies and the like. In most cases, an efficient strategy for library construction involves specifying the enzyme (or chemical) substrate recognition site within or adjacent to the variable nucleotide region of the library so that most members of the library are modified. The substrate recognition site added could be simply a single residue (e.g., serine for phosphorylation) or a complex consensus sequence, as desired.

Conformational constraints, or scaffolding, can also be introduced into the structure of the peptide libraries. A number of motifs from known protein and

array of peptides with many different pharmacological effects.

5 The mu and omega classes (with 6 C's) have 15 possible combinations of disulfide bonds. Usually only one of these conformations is the active ("correct") form. The correct folding of the peptides may be directed by a conserved 40 residue peptide that is cleaved from the N-terminus of the conopeptide to produce the small, mature bioactive peptides that appear in the
10 venom.

 With 2 to 6 variable residues between each pair of C's, there are 125 (5^3) possible framework arrangements for the mu class (2,2,2, to 6,6,6), and 625 (5^4) possible for the omega (2,2,2,2 to 6,6,6,6). Randomizing the
15 identity of the residues within each framework produces 10^{10} to $>10^{30}$ peptides. "Cono-like" peptide libraries are constructed having a conserved disulfide framework, varied numbers of residues in each hypervariable region, and varied identity of those residues. Thus, a sequence
20 for the structural framework for use in the present invention comprises Cys-Cys-Y-Cys-Y-Cys-Cys, or Cys-Y-Cys-Y-Cys-Cys-Y-Cys-Y-Cys, wherein Y is $(NNK)_x$ or $(NNS)_x$, and where N is A, C, G or T, K is G or T, S is G or C, and x is from 2 to 6.

25 Other changes can be introduced to provide residues that contribute to the peptide structure, around which the variable amino acids are encoded by the library members. For example, these residues can provide for α -helices, a helix-turn-helix structure, four helix
30 bundles, etc., as described.

 Another exemplary scaffold structure takes advantage of metal ion binding to conformationally constrain peptide structures. Properly spaced invariant metal ligands (cysteines and histidines) for certain
35 divalent cations (e.g., zinc, cobalt, nickel, cadmium, etc.) can be specifically incorporated into the peptide libraries. Depending on the particular motif specified

desired from the parental peptide ligand. This is useful for screening for any receptor-ligand interaction where one or both members are encoded by a gene, e.g., growth factors, hormones, cytokines and the like, such as
5 insulin, interleukins, insulin-like growth factor, etc.

The peptide-phage libraries of the present invention can also be used to determine the site specificity of enzymes that modify proteins, e.g., the cleavage specificity of a protease. For example, factor X_a cleaves after the sequence Ile-Glu-Gly-Arg. A library
10 of variable region codons as described herein is constructed having the structure: signal sequence--variable region--Tyr-Gly-Gly-Phe-Leu--pIII. Phage from the library are then exposed to factor X_a and then panned
15 on an antibody (e.g., 3E7), which is specific for N-terminally exposed Tyr-Gly-Gly-Phe-Leu. A pre-cleavage panning step with 3E7 can be employed to eliminate clones cleaved by E. coli proteases. Only members of the library with random sequences compatible with cleavage
20 with factor X_a are isolated after panning, which sequences mimic the Ile-Glu-Gly-Arg site.

Another approach to protease substrate identification involves placing the variable region between the carrier protein and a reporter sequence that
25 is used to immobilize the complex (e.g., Tyr-Gly-Gly-Phe-Leu). Libraries are immobilized using a receptor that binds the reporter sequence (e.g., 3E7 antibody). Phage clones having sequences compatible with cleavage are released by treatment with the desired protease.

30 Some peptides, because of their size and/or sequence, may cause severe defects in the infectivity of their carrier phage. This causes a loss of phage from the population during reinfection and amplification following each cycle of panning. To minimize problems
35 associated with defective infectivity, DNA prepared from the eluted phage is transformed into appropriate host cells, such as, e.g., E. coli, preferably by

thereof) to which the desired peptide is sought. The receptor is in one of several forms appropriate for affinity enrichment schemes. In one example the receptor is immobilized on a surface or particle, and the library of phage bearing peptide is then panned on the immobilized receptor generally according to the procedure described below.

A second example of receptor presentation is receptor attached to a recognizable ligand (which may be attached via a tether). A specific example of such a ligand is biotin. The receptor, so modified, is incubated with the library of phage and binding occurs with both reactants in solution. The resulting complexes are then bound to streptavidin (or avidin) through the biotin moiety. The streptavidin may be immobilized on a surface such as a plastic plate or on particles, in which case the complexes (phage/peptide/receptor/biotin/streptavidin) are physically retained; or the streptavidin may be labelled, with a fluorophore, for example, to tag the active phage/peptide for detection and/or isolation by sorting procedures, e.g., on a fluorescence-activated cell sorter.

Phage which express peptides without the desired specificity are removed by washing. The degree and stringency of washing required will be determined for each receptor/peptide of interest. A certain degree of control can be exerted over the binding characteristics of the peptides recovered by adjusting the conditions of the binding incubation and the subsequent washing. The temperature, pH, ionic strength, divalent cation concentration, and the volume and duration of the washing will select for peptides within particular ranges of affinity for the receptor. Selection based on slow dissociation rate, which is usually predictive of high affinity, is the most practical route. This may be done either by continued incubation in the presence of a

the first rounds of screening under conditions to promote multivalent interactions, high stringency washing can be used to greatly reduce the background of non-specifically bound phage. This high avidity step may select a large pool or peptides with a wide range of affinities, including those with relatively low affinity. It may select for specific recognition kernels, such as the Tyr-Gly dipeptide described in the examples below. Subsequent screening under conditions favoring increasingly monovalent interactions and isolation of phage based on a slow dissociation rate may then allow the identification of the highest affinity peptides. Monovalent interactions may be achieved by employing low concentrations of receptor (for example, from about 1 to 100 pM).

It should be noted that, as an aspect of the present invention, determining a dissociation rate for a peptide of interest and the selected receptor molecule under substantially monovalent conditions allows one to extrapolate the binding affinity of the peptide for the receptor. This procedure avoids the necessity and inconvenience of separately determining binding affinities for a selected peptide, which could be especially burdensome if a large number of peptides have been selected.

Once a peptide sequence that imparts some affinity and specificity for the receptor molecule is known, the diversity around this "recognition kernel" may be embellished. For instance, variable peptide regions may be placed on one or both ends of the identified sequence. The known sequence may be identified from the literature, as in the case of Arg-Gly-Asp and the integrin family of receptors, for example, as described in Ruoslahti and Pierschbacher, Science 238:491-497 (1987), or may be derived from early rounds of panning in the context of the present invention.

others, Zacher et al., Gene 9:127-140 (1980), Smith et al., Science 228:1315-1317 (1985) and Parmley and Smith, Gene 73:305-318 (1988).

5 Construction of Vector fAFF1

 A filamentous bacteriophage vector was constructed from the tetracycline resistance transducing vector fdTet, described in Zacher et al., supra. The vector, designated fAFF1, was designed to provide many choices in the size and location of the peptides expressed fused to the pIII bacteriophage coat protein. pIII is made as a preprotein with an 18 amino acid leader sequence that directs pIII to the inner membrane of the bacterial host cell before it becomes assembled into an intact phage particle (Goldsmith and Konigsberg, Biochem. 16:2686-2694 (1977) and Boeke and Model, Proc. Natl. Acad. Sci. USA 79:5200-5204 (1982) incorporated herein by reference). As explained further below, a peptide library was constructed by cloning an oligonucleotide of the structure shown in Fig. 1B to place the variable hexapeptide region at the N-terminus of the processed protein. These first six residues are followed by two glycines and then the normal sequence of pIII. The library consists of about 3×10^8 independent recombinants.

 A cloning site, consisting of two non-complementary BstXI sites, was engineered into the 5'-region of gene III. As shown in Fig. 1A, two non-complementary BstXI sites flank the region encoding amino acids surrounding the signal peptidase site (the N-terminus of the mature pIII). fAFF1 also has a -1 frameshift mutation in pIII that results in non-infective phage. By removing the BstXI fragment and inserting an oligonucleotide of the appropriate structure, (a) portions of the removed sequence can be precisely reconstructed (the correct signal peptide site, for example,) (b) one or more additional amino acids may be

annealing to two "half-site" oligonucleotides, ON-28 (5'-GGA GTG AGA GTA GA-3') and ON-29 (5'-CTT TCA ACA GT-3'), which are complementary to the 5'- and 3'- portions of ON-49, respectively. "Half-site" oligonucleotides anneal to the 5'- and 3'- ends of oligonucleotide ON-49 to form appropriate BstXI cohesive ends. This left the appropriate BstXI site exposed without the need to digest with BstXI, thus avoiding the cutting of any BstXI sites that might have appeared in the variable region. The vector fAFF1 (100 μ g) was digested to completion with BstXI, heat inactivated at 65°C, and ethanol precipitated twice in the presence of 2 M ammonium acetate. Oligonucleotides were phosphorylated with T4 kinase, and annealed in 20 mM Tris-HCl, pH 7.5, 2 mM MgCl₂, 50 mM NaCl, by mixing 1.5 μ g ON-28, 1.2 μ g ON-29, and 0.5 μ g ON-49 with 20 μ g BstXI-digested fAFF1 RF DNA, heating to 65°C for 5 minutes and allowing the mixture to cool slowly to room temperature. This represented an approximate molar ratio of 1:5:100:100 (fAFF1 vector: ON-49: ON-28: ON-29). The annealed structure is then ligated to BstXI-cut fAFF1 RF DNA to produce a double-stranded circular molecule with a small, single stranded gap. These molecules may be transformed into host cells. The annealed DNA was ligated in 20 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 2 mM DTT, 1 mM ATP, by the addition of 20 units of T4 DNA ligase and incubated overnight at 15°C.

Alternatively, before transformation, the gap may be filled-in under conditions disfavoring secondary structure in the variable region.. In some experiments the gapped circular structure created by this ligation was filled in with T4 DNA polymerase in the presence of ligase and dNTPs (400 μ M each) to produce a covalently closed, double-stranded molecule (Kunkel et al., supra). The ligated DNA was ethanol precipitated in the presence of 0.3 M sodium acetate, resuspended in water, and transformed by electroporation into MC1061. Five electro-transformations, each containing 80 μ l of cells

of Parmley and Smith, supra. A 60 x 15 mm polystyrene petri plate was coated with 1 ml of streptavidin solution (1 mg/ml streptavidin in 0.1 M NaHCO₃, pH 8.6, 0.02% NaN₃) and incubated overnight at 4°C. The streptavidin solution was removed the following day. The plate was filled with 10 ml blocking solution (30 mg/ml BSA, 3 µg/ml streptavidin in 0.1 M NaHCO₃, pH 9.2, 0.02% NaN₃) and incubated for two hours at room temperature. Biotinylated goat anti-mouse IgG (2 µg) was added to the antibody-reacted phage library and incubated for two hours at 4°C. Immediately before panning, the blocking solution was removed from the streptavidin-coated plate, and the plate was washed 3 times with TBS/0.05% Tween 20. The antibody-reacted phage library was then added to the plate and incubated for 30 min. at room temperature. The phage solution was removed and the plate was washed ten times with 10 ml TBS/0.05% Tween 20 over a period of 60 min. at room temperature. Adherent phage were removed by adding 800 µl of elution buffer (1 mg/ml BSA in 0.1 N HCl adjusted to pH 2.2 with glycine) to the petri plate and incubating for 10 min. to dissociate the immune complexes. The eluate was removed, neutralized by addition of 45 µl of 2 M Tris base, and used to infect log phase E. coli K91 cells.

The infected cells were then plated on LB agar plates containing tetracycline (20 µg/ml), and grown overnight at 37°C. Phage were isolated from these plates as described above and the affinity purification process was repeated for two more rounds. After each round of panning and amplification, DNA of phage from several thousand colonies was pooled and sequenced to estimate the diversity in the cloning site. In the first two positions of each codon, bands of about the same intensity appeared in each lane, indicating the expected distribution of bases in these positions. In the third position of each codon the G band was somewhat more intense than the T band.

Table 1: Nucleotide Distribution in the Diversity Region of Infectious Phage Randomly Selected from the Library.

5	Frequency of each base by position in codon (%)			
	N	N	K	
10	G	31	27	59
	A	22	22	<1
	T	25	26	39
	C	22	24	1

15

20

In Fig. 2, the amino acid sequences are listed for the peptides encoded by the oligonucleotide inserts of a sample of randomly selected, infectious phage. The amino acid content of the expressed peptides from the 52 randomly selected infectious phage appears in Table 2.

As shown in Table 2, the ratio of the observed occurrence of each amino acid to that expected on the basis of codon frequency ranges from about 0.5 to 2, consistent with a random distribution of sequences.

Constructing a library of peptides displayed on the N-terminus of processed pIII necessarily alters amino acids in the vicinity of the signal peptidase cleavage site. Certain changes in the corresponding region of the major coat protein, pVIII, have been shown to reduce processing efficiency, slowing or preventing the incorporation of pVIII to virions. If pIII were similarly affected, the diversity of peptides contained in the library would be reduced. The finding that most amino acids appear at each position of the variable peptides of randomly selected phage indicates that processing defects do not impose severe constraints on the diversity of the library.

Isolation and sequencing of phage having high avidity for anti-b-endorphin antibody.

Monoclonal antibody 3E7 binds to B-endorphin and, like the δ -opioid receptor, recognizes the N-terminal portion of the protein (Tyr-Gly-Gly-Phe), which is present on most natural opioid peptides. The antibody also binds tightly to leu- and met-enkephalin (YGGFL, YGGFM), and a variety of related opioid peptides (Meo et al., Proc. Natl. Acad. Sci. USA 80:4084-4088 (1983), Herz et al., Life Sciences 31:1721-1724 (1982), and Gramsch et al., J. Neurochem. 40:1220-1226 (1983). The N-terminal hexapeptide library was screened against 3E7 by carrying out three rounds of panning, elution, and amplification. The recoveries of phage from this process are shown in Table 3. In each round the proportion of phage adsorbed to the antibody increased by about 100-fold, and in the last round, over 30% of the input phage were recovered. These results indicated that phage were preferentially enriched in each panning step.

more often than would be expected by chance. The fourth position is occupied primarily by the large aromatic residues Trp and Phe (together 50%), and the bulky hydrophobic residues Leu and Ile (an additional 45%). The fifth and sixth positions contain essentially random distributions of amino acids, with only alanine appearing at slightly greater than chance in position five.

Table 4: Distribution of Amino Acids in the Diversity Peptide of 51 Phage Selected by Panning With Anti-endorphin Antibody

Residue Enrichment ^(a) Position	Amino Acid	Nominal Frequency	Observed Frequency	Ratio
1	Y	.031	1.00	33
2	G A,S	.062	0.94	16 <1
3	G W S A N	.062 .031 .093 .062 .031	0.31 0.10 0.21 0.12 0.06	5 3 2 2 2
	D,E,F,K, L,M,P,T,V			<1
4	W F L I A,G,M	.031 .031 .093 .031	0.31 0.19 0.35 0.10	10 6 4 3 <1

a. Observed frequency divided by nominal frequency.

EXAMPLE II

Binding Affinities of Peptides
for Receptor Monoclonal Antibody 3E7

Shown in Table 5 are the IC₅₀ for the six peptides which were identified by the phage panning method and chemically synthesized. Under the conditions of the radioimmunoassay (30 pM [¹²⁵I]b-endorphin; 20% tracer bound; 18 hr. incubation), the IC₅₀ should be very close to the dissociation constant (K_d) for the peptide. The peptides are all relatively low affinity compared to YGGFL, with IC₅₀'s ranging from 0.35 to 8.3 μ M.

Table 5: Relative affinities of peptides for 3E7 antibody determined by solution radioimmunoassay.^a

Peptide	N	IC ₅₀ (μ M)	Affinity Relative to YGGFL
YGGFL	(6)	0.0071 (0.0054, 0.0093)	1
YGGF	(3)	0.19 (0.093, 0.38)	0.037
YGGL	(3)	3.8 (2.1, 6.6)	0.0018
YGFL	(3)	28 (17, 47)	0.00025
YGG	(2)	>1000	<0.0000071
GGFL	(2)	>1000	<0.0000071
GGF	(2)	>1000	<0.0000071
GFL	(2)	>1000	<0.0000071
YGFWGM	(3)	0.35 (0.19, 0.63)	0.020
YGPFWs	(3)	1.9 (1.3, 2.8)	0.0037
YGGFPD	(3)	2.3 (1.4, 3.7)	0.0031
YGGWAG	(3)	7.8 (6.0, 10)	0.00091
YGNWTY	(3)	7.8 (4.0, 15)	0.00091
YAGFAQ	(3)	8.3 (3.8, 18)	0.00086

^a = Data are geometric means and 95% confidence intervals (calculated from S.E.M. of log IC₅₀) from the number (N) of independent determinations indicated.

The data indicate that although the phage panning method is highly specific in that no unrelated peptides were selected, the procedure apparently does not discriminate between those of moderate (μ M K_d) and high (nM K_d) affinity. The six peptides chosen from among the 51 clones that were sequenced were only a small subset of those which were selected by three rounds of panning. Based on their structural diversity, the phage library

purification, the specific activity of the [¹²⁵]FAB was approximately 15 μ Ci/ μ g.

Antisera were raised against phage particles lacking pIII fAFF1, which, as described above, contains a frameshift in the 5' end of gene III and is produced as non-infective polyphage. Cells from a two liter culture of E. coli K91 were removed by centrifugation and media was mixed with 400 ml of 20% PEG in 0.5M NaCl. After incubation for 1 hr at 4 C. precipitated phage were isolated by centrifugation at 8500 rpm. The pellet was resuspended in TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) and then ultracentrifuged in a SW50 rotor at a 42,000 rpm for 3 hrs. The resulting pellet was resuspended in water and the concentration of phage was estimated according to the method of Day, J. Mol. Biol. 39:265 (1969).

Three rabbits were injected intramuscularly with 0.5 mg of phage in Freund's complete adjuvant and then boosted with 0.25 mg of phage in incomplete adjuvant at 3 week intervals. The titer of the sera was measured with an ELISA using phage immobilized in Immulon 2 microtiter wells as described above. All rabbits produced high titer sera after the second boost. Sera collected after the third boost from one of the rabbits was used for the assays.

Antibodies reacting with phage were affinity purified as follows. Phage expressing native pIII (Fd-tet) from a two liter culture were isolated (described above) and added to 20 ml of sera that was diluted 4-fold with PBS (10 mM sodium phosphate, 150 mM NaCl, pH 7.4). After incubation for 2 hr at room temperature, phage/antibody complex was isolated by centrifugation for 1 hr at 120,000 x g. The pellet was washed with 10 ml of PBS and centrifuged again. The final pellet was resuspended in 10 ml of 100 mM sodium acetate buffer pH 2.5 and incubated for 10 min. at room temp. The sample was subjected to same centrifugation and the resulting supernatant was neutralized with NaOH. IgG was then

differ when IgG and Fab were used. In combination with the data on monovalent dissociation rates of these peptides (see below), this suggests that antibody binding sites for both IgG and Fab are in sufficient proximity to one another to allow simultaneous binding of more than one of the peptides expressed by each phage particle.

The phage sandwich assay can also be used to determine the specificity and competitive nature of the interaction of peptide-bearing phage with immobilized antibody. In practice, an important aspect of the use of peptide on phage libraries is the characterization of individual phage isolates after sequential rounds of the affinity purification. Isolated phage may bind to other components found on an immobilizing surface, or may bind to the protein target at sites other than the active site. Using the phage sandwich assay, the binding of YGGFL- and YAGFAQ-phage was shown to be specific for the antibody and the interaction of the phage with antibody could be blocked by free YGGFL peptide.

Shown in Fig. 6 are the results of tests on the effect of Fab concentration and wash time on the recoveries of YGGFL- and YAGFAQ-phage. Microtiter wells were coated with streptavidin as described above. 10^{11} infectious phage particles bearing the peptides YGGFL or YAGFAQ were incubated overnight at 4°C with either 50 μ l of 5nM or 50 pM biotinylated Fab. Aliquots were then added to different microtiter wells and incubated for 1 hr. at room temperature. All the wells were washed quickly with TBS/0.05% Tween 20, with the last 200 μ l wash being left in the well. At various times thereafter, wells washed quickly with TBS/0.05% Tween and the phage remaining bound were eluted with 0.1 M HCl (pH adjusted to 2.2 with glycine) and quantitated by titering as described above.

The results indicate that low Fab concentration (50pM) and dissociation times greater than 30 minutes allowed the selective recovery of phage bearing the

only one repeat (the nucleotide sequence was also identical).

TABLE 6

	<u>Dissociation t_{1/2}</u>	<u>Equilibrium IC₅₀</u>
	(minutes)	(nM)
<u>Control Peptides</u>		
YGGFL *	18.2	6.6 ± 3.5
YGFWGM*	0.25	350
YAGFAQ*	**	8300
<u>Peptides isolated with 50pM Fab</u>		
YGAFAQG	18.9	27 ± 2.0
YGGFLT	17.9	
YGYWSL	15.6	
YGAFMQ	13.7	13 ± 4.9
YGAFFQ	13.4	
YGAFFK	9.1	59 ± 22
YGFWSN	7.4	
YGAFGG	5.0	
YGGFGF	4.7	65 ± 18
YGVFSR	2.8	
YGGLSM	0.96	
YGTFLN	0.75	470 ± 140
YGGLVR	0.50	
YGSFSL	0.43	
YGAWYT	**	1600 ± 300
YGRFFH	**	
YGGLRH	**	
YGSFMA	**	
<u>YGGFSP</u>	<u>**</u>	

** indicates that initial binding was not detected

Determination of the dissociation of [¹²⁵I]Fab from fusion phage clones.

An assay employing the anti-phage antisera was developed to determine the rate of dissociation of [¹²⁵I]Fab from individual phage isolates. Individual

requirements for selection using low Fab concentrations were established.

Several peptides corresponding to those phage clones with differing dissociation rates were chemically synthesized and their potencies were determined in a solution competition assay. The $t_{1/2}$ values correlated with the IC_{50} of the corresponding free peptide. Under the conditions of the competition assay (low concentration of tracer, <20% bound tracer, 18 hr. incubation), the IC_{50} should approximate the K_d . For phage bearing peptides with K_d s greater than 500 nM, specific binding was not detected under these monovalent assay conditions.

EXAMPLE IV

Conotoxin Peptide Libraries Having Conserved Disulfide Frameworks.

A conotoxin peptide library is prepared as generally described above, by synthesizing oligonucleotides containing degenerate codons of the NNK (or NNS) motif. Here N is equimolar A,C,G, or T, and K is equimolar G or T (S=G or C). This motif codes for all 20 amino acids at each locus in the hypervariable regions. (Alternatively, the degenerate portion can be assembled by the condensation of 20 activated trinucleotides, one for each amino acid.) The six cysteine codons are preserved to produce the characteristic conotoxin frameworks.

To sample additional diversity in the peptide libraries, the number of residues between the Cys's is varied. This is accomplished as follows:

(1) Five separate oligonucleotide synthesis columns are prepared with the first nucleotide immobilized on resin. (2) The common regions of the 3' end of the oligonucleotides is synthesized (all columns

phage in vitro is employed, followed by mild oxidation to form most of the conformations. Mild reduction/oxidation can be accomplished by treatment with 0.2 to 5 mM DTT followed by extensive dialysis to non-reducing conditions. A regenerable, immobilized lipoic acid column to rapidly pass the peptide-bearing particles over can also be used.

The possibility of promiscuous binding of Cys residues in the peptide binding to other proteins can also be minimized by mild reduction and oxidation, or can be avoided by re-engineering the fusion protein by site-directed mutagenesis to remove the Cys residues.

Peptides with the conotoxin framework can be expressed in several types of libraries as described herein. For example, the peptides can be 1) expressed in an N-terminal library in phage fAFF1; 2) expressed internally, fused to pIII at or near the N-terminus, displacing the degenerate peptides 2 to 10 or more residues from the cleavage point to circumvent processing problems; 3) expressed in a carboxy terminal exposed library (as many of the conotoxins are C-terminally amidated, residues with amino side chains can be added to the C-terminal end of the peptides, or the peptide library, can be amidated in vitro); and 4) the putative 40 residue "folding peptide" can be installed upstream of degenerate peptides displayed in the C-terminally exposed configuration.

This general format for using the secondary framework structure of conotoxins can also be applied to other peptide families with biological activities as a basis for designing and constructing peptide expression/screening libraries in accordance with the present invention.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will

WHAT IS CLAIMED IS:

1. A method for identifying peptides of interest which bind to a preselected receptor, comprising:

5 transforming host cells with a bacteriophage expression vector which comprises an oligonucleotide library of at least about 10^6 members which encode peptides, wherein a library member is joined in reading frame to the 5' region of a nucleotide sequence encoding an outer structural protein of the bacteriophage;

10 cultivating the transformed host cells under conditions suitable for expression and assembly of bacteriophage;

15 contacting bacteriophage that express the peptides to the preselected receptor under conditions conducive to specific peptide-receptor binding; and

selecting bacteriophage which bind to the receptor and therefrom identifying the peptides of interest.

20 2. The method of claim 1, further comprising the step of determining the nucleotide sequence encoding the peptide of interest in the selected bacteriophage.

25 3. The method of claim 1, wherein the selected bacteriophage are propagated and the contacting and selecting steps are repeated to enrich for bacteriophage which express the peptides of interest.

30 4. The method of claim 3, wherein the valency of the specific peptide-receptor binding interaction is reduced in subsequent repetitions of the contacting, selecting and propagating steps to enrich for peptides of higher binding affinity.

35 5. The method of claim 1, wherein the bacteriophage expressing peptides and receptor are present at concentrations that produce a substantially monovalent

15. The method of claim 14, wherein the bacteriophage is fd or a derivative thereof.

5 16. The method of claim 15, wherein the outer bacteriophage protein is a coat protein.

17. The method of claim 16, wherein the coat protein of the fd bacteriophage is pIII.

10 18. The method of claim 1, wherein the oligonucleotide library comprises a series of codons encoding a random collection of amino acids.

15 19. The method of claim 18, wherein the codons encoding the collection of amino acids are represented by $(NNK)_x$ or $(NNS)_x$, where N is A, C, G or T, K is G or T, S is G or C, and x is from 5 to 8.

20 20. The method of claim 19, wherein the series of codons encoding the random collection of amino acids of the oligonucleotide library member encodes a hexapeptide.

25 21. The method of claim 19, wherein x is 8 and the recombinant bacteriophage screened in the selecting step represents up to about 10% of the possible octapeptides.

30 22. The method of claim 18, wherein the oligonucleotide library member further encodes at least one spacer residue.

23. The method of claim 22, wherein a spacer residue comprises Gly.

35 24. The method of claim 23, wherein the spacer comprises Gly-Gly.

33. The method of claim 1, wherein the host cells are transformed by electroporation.

5 34. The method of claim 1, wherein the oligonucleotide library comprises at least about 10^8 members.

10 35. The method of claim 1, wherein the oligonucleotide library members are inserted in the bacteriophage expression vector so that the N-terminus of the processed bacteriophage outer protein is the first residue of the peptide.

15 36. The method of claim 1, wherein the bacteriophage protein is a preprotein which is processed by the host cell to leave the peptide encoded by an oligonucleotide library member exposed at the N-terminus of the mature outer structural protein.

20 37. The method according to claim 36, wherein the peptide comprises spacer amino acid residues are encoded by the oligonucleotide library members between the N-terminus of the mature outer protein and the C-terminus of the peptide.

25 38. A method for identifying peptides of interest which bind to a preselected receptor, comprising:
transforming host cells with a bacteriophage
expression vector which comprises an oligonucleotide
30 library which encodes peptides, wherein a library member is joined in reading frame with a nucleotide sequence to encode a fusion protein, wherein the library member represents the 5' member of the fusion protein and the 3' member comprises at least a portion of an outer
35 structural protein of the bacteriophage;

cultivating the transformed cell under conditions suitable for expression and assembly of bacteriophage;

library member from a randomly generated mixture of oligonucleotides.

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C	M	L	O	R	L	C	R	G	N	S	G	D	W	V	G	G	A
M	S	R	K	L	F	E	R	A	A	S	V	T	A	M	Q	P	G
L	W	A	G	H	E	L	W	A	V	F	M	L	D	L	K	R	L
V	G	I	T	Q	G	G	V	G	A	L	N	V	R	N	S	M	G
K	T	S	R	G	E	D	S	N	P	L	C	R	V	C	N	K	T
Q	K	T	G	R	Q	A	K	H	M	W	S	G	N	M	A	H	Q
T	L	S	L	F	A	A	V	Q	R	R	F	D	S	F	C	R	F
S	V	S	A	R	A	L	W	S	L	G	Q	A	V	L	M	Q	W
A	G	A	R	L	G	A	E	K	P	R	G	K	H	Y	Q	S	F
K	A	R	L	F	A	M	R	L	V	S	R	S	R	K	S	V	D
L	A	F	L	P	K	V	C	S	L	H	A	G	S	S	R	V	D
D	L	S	P	V	S	L	R	C	S	L	G	E	Y	S	C	R	S
D	S	E	R	L	C	R	A	R	T	W	F	S	A	P	S	T	R
R	A	A	T	L	R	S	I	W	L	G	S	Y	E	P	Q	S	K
I	T	A	T	P	G	L	V	A	G	L	L	S	I	G	F	C	C
I	S	N	D	L	S	G	G	G	F	T	M	V	C	P	Q	F	C

FIG. 2.

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YGG LGL	YGS LVL	YGA LGG	YGW WGL	YGL WQS
YGG LGI	YGS LVO	YGA LSW	YGW WLT	YGF WGM
YGG LGR	YGS LVR	YGA LDT	YGW LAT	YGK WSG
YGG LNV	YGS LAD	YGA LEL	YGW ANK	YGP FWS
YGG LRA	YGS LLS		YGN WTY	YGE FVL
YGG LEM	YGS LNG	YGA IGF	YGN FAD	YGD FAF
	YGS LYE		YGN FPA	
YGG IAS	YGS WAS	YGA WTR		YAW GWG
YGG IAV	YGS WAS		YGT FIL	YAG FAQ
YGG IRP	YGS WQA		YGT WST	YSM FKE
YGG IRP				
YGG WAG	YGS FLH		YGV WAS	
YGG WGP			YGV WWR	
YGG WSS				
YGG MKV				
YGG FPD				

FIG. 4.

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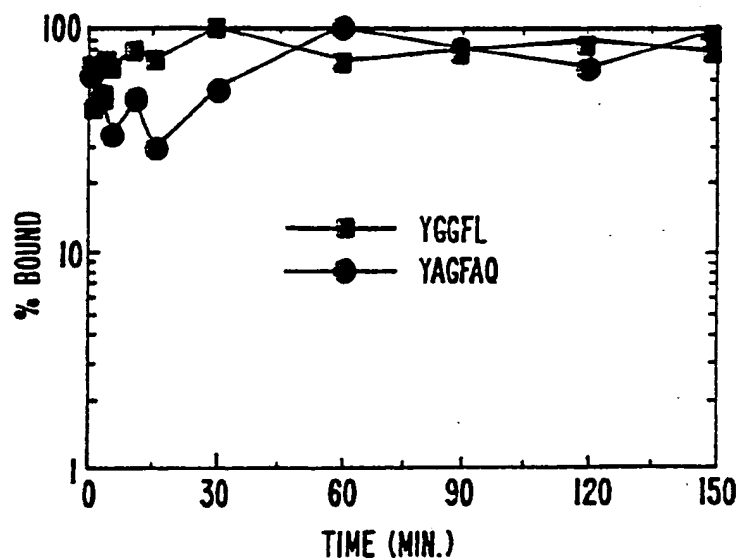


FIG. 6A.

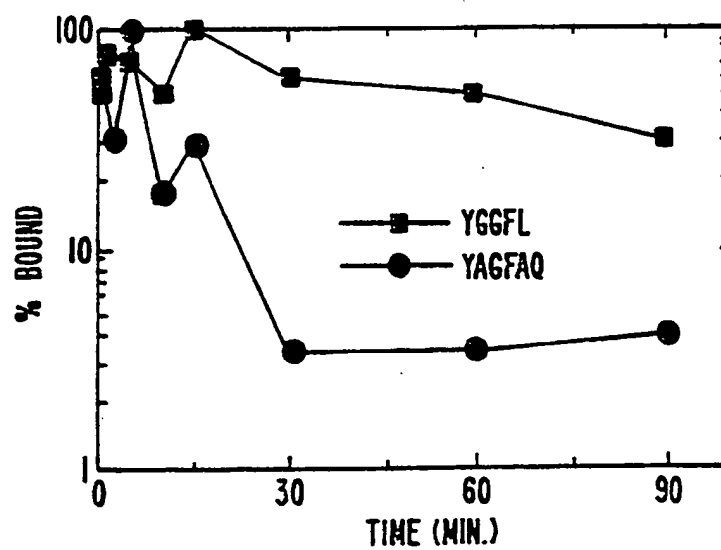


FIG. 6B.

INTERNATIONAL SEARCH REPORT

International Application No. **PCT/US91/04384**

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12Q 1/70; A61K 37/02

U.S.CL.: 435/5; 530/300

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷

Classification System	Classification Symbols
US.	435/5,69.1,71.1; 530/300;935/

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁸

APS Dialog

III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹

Category [*]	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
N-P	Proceedings of the National Academy of Sciences. Volume 87. issued August 1990. Cwirla et al., "peptides on Phage: A vast Library of Peptides for Identifying Ligands," pages 6378-6382. see entire document.	1-40
A	Gene Volume 44. issued 1986. Oliphant et al., "Cloning of Random-Sequence Oligodeoxynucleotides," pages 177-183. see entire document.	1-40

^{*} Special categories of cited documents: ¹⁰

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATE

Date of the Actual Completion of the International Search

18 October 1991

International Searching Authority

ISA/US

Date of Mailing of this International Search Report

07 NOV 1991

Signature of Authorized Officer

Donna C. Wortman